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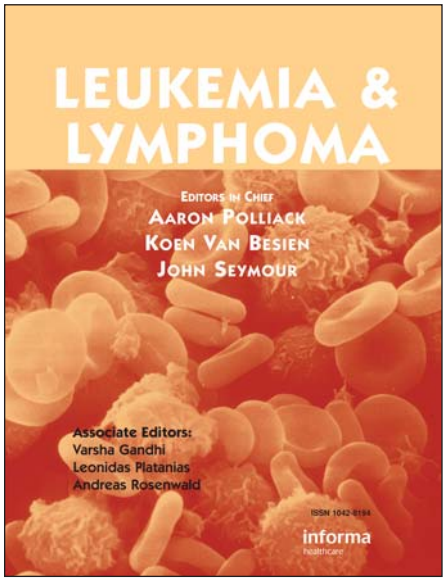

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Short title: IRF in DLBCL

ABSTRACT

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In a discovery driven approach we searched for new translocation partners of the IGH in DLBCL by long distance inverse (LDI) PCR and Sanger sequencing. A t(14;16)(q32.33;q24.1) *IGH/IRF8* was detected in a CD5+ de novo DLBCL, confirmed by translocation specific PCR and FISH analysis. No further IRF8 aberration could be identified neither by LDI-PCR in additional five CD5+ DLBCL nor by FISH on 78 FFPE biopsies. Subsequent screening for IRF8 by immunohistochemistry revealed IRF8 expression in 18/78 (23%) correlating with a GCB type of DLBCL. This hitherto unknown translocation t(14;16)(q32.33;q24.1) is alike to represent the initiator of a multistep lymphomagenesis in a CD5+ de novo DLBCL.

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Introduction

Chromosomal translocations involving the immunoglobulin loci represent frequent oncogenic events in B-cell lymphoma development. They are generally initiated by accidental double strand breaks of oncogenes and by regular double strand cleavage occurring in conjunction with the physiological V(D)J rearrangement or class switch recombination processes during B-cell maturation. The translocation is finally the result of an erroneous double strand repair linking the wrong ends of the disrupted DNAs. Consequently, the incoming partner gene comes under the control of the *IGH* locus and is thereby de-regulated [1,2]. As described above, *IGH* gens undergo molecular rearrangements in an orchestrated way dependent on their maturation state. Therefore, the knowledge of the alignment of functional rearrangements of the *IGH* allows to draw conclusions at which maturation step a malignant transformation occured [1,3]. *LDI-PCR* is a three step method, which allows the rapid amplification of un-characterized DNA sequences (such as unknown translocation partners) neighbouring a known genomic region (such as IGH). The PCR template is a self-ligated circularized DNA, reason for, the primer set of the known nucleotide sequence is in opposite orientation. Together with a thermostable DNA polymerase, this method was initially used for rapid cloning of the IGH joining region [4].

Hence, the aim of our study was to find new translocations involved in the pathogenesis of DLBCL. For this purpose, we established an LDI-PCR approach for an initial screening for novel IGH-translocation partners in fresh frozen tissues, and we extended the study on one particular candidate gene by analysing a larger cohort of lymphomas in a tissue micro array (TMA) setting employing immunohistochemistry and interphase FISH techniques.

Materials and Methods

Patients and Specimen Selection

All histological diagnosis were initially reviewed on whole sections according to the current WHO classification and further sub-classified according to the Hans' classifier into GCB and non-GCB type [5,6]. The investigation of the target gene by LDI-PCR was carried out on fresh frozen biopsies of a total of six patients with a CD5+ DLBCL, four of them with a CD5+ de novo DLBCL (including the index patient) and one each with Richter transformation and transformed lymphoplasmocytic lymphoma/M. Waldenström. Additionally, formalin fixed, paraffin embedded biopsies of 78 patients diagnosed for DLBCL between 1990 and 1999 were investigated on a TMA. The index patient was a female of 73 years of age, who died 24 days after diagnosis of lymphoma.

This study was done in accordance with Swiss laws and approved by the official authorities of the ethical committee of the Canton Zurich (StV26-2006).

LDI-PCR

DNA was isolated from snap-frozen tumour specimens using standard protocols [7]. In a first step 500-800 ng of high quality DNA was digested with the restriction enzymes BglII, HindIII, XbaI or SphI, each cleaving near the joining or switch region of the *IGH* gene, respectively (**Fig1**). The digested DNA was purified by QiAquick gel extraction kit (Qiagen) followed by a ligation step. In a total reaction volume of 50 µl, 5.0 µl QiAquick eluate of the restriction digest was added to 5.0 µl X ligase buffer (Roche, Switzerland) and 2U high concentration ligase (0.2 µl), and ligated at 16°C over night. Long range PCR was carried out using *0.2 µM* primer concentration with cycling conditions as published previously [7]. For long distance inverse PCR inversely oriented primer pairs located in the vicinity of Eµ-enhancer or the switchµ region were employed. PCR products were subjected to electrophoresis on 0.5% agarose gels, bands were excised, purified with QiAquick gel extraction kit and directly sequenced. The sequence was compared to the Genbank and IMGT/Quest databases using BLASTN. The new translocation was verified by translocation specific PCR on different starting materials including snap frozen and paraffin embedded tissue of the index patient IgVH mutation analysis was carried out as published [8]. In addition to framework FR3 and FR2a primers combined with a consensus JH primer, family specific primers for VH4L and the above consensus primer were applied for the determination of the mutational status. For primers see (**Table1**).

FISH

The FISH probe for IRF8 was biotin-labeled by nick translation. Detection was done with tetramethylrhodamine-5-(and-6)-isothiocyanate (5(6)-TRITC) conjugated avidin and included an amplification step with biotinyliated goat anti-avidin (BioGAA)[9]. The corresponding BAC clone had been verified beforehand by PCR with primers specific for IRF8 and by direct sequencing of a 300 bp long PCR fragment. Customized FITC labelled IGH probes (Chrombios, Raubling, Germany) were admixed to the IRF8 probe. In addition, commercially available break-apart LSI probes for IGH, BCL-2, BCL-6, MYC and a fusion probe for IGH/CCND1 (Abbott molecular) were applied according to the manufacturer's instruction. For primers and BAC clones see (**Table 1**).

Immunohistochemistry

Immunohistochmistry for lymphoma classification was performed as published recently [10]. A rabbit polyclonal antibody (H-70; Santa Cruz Biotechnology) raised against a recombinant C-terminal fragment of the ICSBP-1 protein (amino acids 357-426) was used. All techniques were carried out on an automated Ventana instrument using a diaminobenzidine immunoperoxidase detection kit (Roche's Ventana Medical Systems, Basel, Switzerland). Double staining for DAB was carried out with an Ultra View Universal AP Red Detection Kit (Roche's Ventana Medical Systems, Basel, Switzerland). The staining pattern in reactive tonsils served as reference for the antibody quality (**Fig2**).

An immunoreactivity score according to Bittinger and Brochhausen was calculated by multiplying the staining intensity (IS: 0-3) with the percentage of positive cells (PP: 0-100%) [11]. Score 1 (score 1 or <30% IS 1-2) and score 2

(score 1-2 or <30% IS 2) were regarded as negative, whereas score 3 (score 2 or <30% IS 3) and score 4 (score 3 or > 50%) were regarded as positive staining.

Statistics

Correlation between IRF8, GCB and non-GCB type of DLBCL were carried out using a two-sided Fisher's exact test. P-values <0.05 were considered significant. Overall survival analysis was estimated with the Kaplan-Meier method. OS was determined from the date of histological lymphoma diagnosis to the date of disease related death of any cause or last follow-up. Statistical analysis was performed using the software package SPSS® (Version 12.0.1 for Windows®, ©SPSS Inc., Chicago, USA).

Results

In a de novo CD5+ DLBCL a translocation joining the JH2 region of the IGH gene on chromosome 14q32.33 with a sequence localised about 500 bp upstream of the IRF8 gene on chromosome 16q24.1 was identified by LDI-PCR and sequencing. The breakpoint was subsequently verified by standard PCR of DNA-extracts of fresh frozen as well as of paraffin embedded tissues from the index patient (**Fig1**). Interphase FISH analysis showed a split signal of one allele of the IGH gene with an IGH break-apart probe and a fusion of the IGH- and IRF8-signal with the double colour single fusion approach in the same lymphoma (**Fig2E-F**). Moreover, we detected somatic hyper-mutations in the variable region of the second IGH allele, characteristic of a germinal centre (GC) reaction. No indications for the presence of breaks involving the CCND1, BCL-2, BCL-6 or MYC gene could be found neither by LDI PCR nor by conventional interphase FISH. Immunohistochemistry revealed a strong IRF8 protein expression (score 4) in the CD20 positive tumour cells (**Fig3C**).

In the tissue microarray study, 18/78 (23%) DLBCL showed evidence for an overexpression of IRF8 (immunohistochemical staining score 3 and 4) (**Fig2**), significantly associating with a GCB-phenotype (CD10+/Bcl-6+/IRF4-) (p=0.012), but inversely correlating with an IRF4 expression (p=0.001). Among the 78 patients represented on the TMA, there was one with a CD5+ positive, non-GCB type DLBCL showing a faint staining (score 2) for IRF8 which was regarded as negative. Survival data of 32 out of 78 patients were available, but no correlation of IRF8 expression with overall survival could be found (p=0.385). No further t(14q32.33;16q24.1) translocation could be identified by LDI-PCR in the remaining five frozen tissues of CD5+ DLBCL nor in the 78 formalin-fixed DLBCL biopsies analysed by FISH on the TMA.

Discussion

We describe a hitherto unknown translocation t(14q32.33;16q24.1) joining the IGH and the IRF8 loci in a de novo CD5+ DLBCL. As shown by sequence analysis of the LDI-PCR product, and underscored by the IGH FISH analysis, the translocation occurred on one allele most likely during the VDJ

rearrangement process in the bone marrow. Apparently, the RAG1/RAG2 recombination enzyme complex cleaved the JH region of the IGH gene regularly. In contrast, the breakpoint immediately upstream of the IRF8 may have been caused accidentally without participation of the RAG-enzymes as no cryptic recombination signal sequence (RSS) could be identified in its vicinity. Somatic hyper-mutations in the variable region of the second normally rearranged IGH allele, which is characteristic of a germinal centre (GC) reaction, indicates further progression of B-cell maturation. Additionally, the variable expression of IRF4/MUM1 in some, but not all of the lymphoma cells suggests a transit through the germinal centre (GC) after having acquired the somatic mutations.

The translocation partner gene encodes interferon (IFN) consensus sequence binding protein-1 (ICSBP-1) also designated as IFN regulatory factor-8 (IRF8). This protein belongs to the family of IRF transcription factors. IRF8 regulates multiple stages of immune response, particularly through macrophage function [12,13]. Furthermore, the related IRF transcription factors IRF4/MUM1 and IRF8 seem to be required for the immunoglobulin light chain, but not the heavy chain gene rearrangement in mice [14]. Since the IRF8 can act as a transcriptional repressor as well as an activator, its ultimate function is defined by the interacting partner proteins (reviewed in [14,15]).

Here, we confirm the result of a recent study, showing IRF8 expression associated with a GC phenotype [16]. The fact, that we were not able to associate IRF8 expression with survival, in spite of its association with a GCB-type of DLBCL, might be explained with the very heterogeneous treatment modalities used during recruitment time and/or in the classifier applied. IRF8 expression in nearly one fourth of DLBCL (18/78, 23%) speaks in favour of a hazardous association with the index CD5+ DLBCL, but not for a general pattern in primary or secondary CD5+ DLBCL. Accordingly to these observations, although easily applicable, IRF8 appears not bear prognostic potential in DLBCL.

However, by demonstrating a specific IRF8/IGH translocation, we provide strong evidence that a deregulated expression of IRF8 participates in the oncogenic process. In the lymphoma cells of our index patient, we hypothesize that IRF8 had come under the control of the IGH enhancers similar to other genes erroneously linked to the IGH-locus. Despite the over-expression of IRF8 observed in about one fourth of DLBCLs, mechanisms different than a gene activation via the t(14q32.33;16q24.1) translocation may be responsible. One could hypothesize that the recently described IRF8 mutations described in a few DLBCLs (in 4 out of 73 (5.47%) primary DLBCL and in one cell line) may be linked to an altered expression in at least some of the cases in our patients' collective17. Yet, in our index lymphoma we did not identify any mutation in the coding sequences of the IRF8 gene. Moreover, in contrast to newly described translocations activating IRF4 in GC derived lymphomas in children and young adults, IRF8 seems to be a rare IGH-translocation partner in adults 18. Anticipating a potential heterogeneity among the breakpoint localizations near the IRF8 gene in different DLBCL patients, we opted for a single fusion FISH approach for the screening for similar t(14;16) translocations in the TMA, while accepting a slight reduction in sensitivity in comparison to a break apart assay.

Translocations with IGH involvement are generally frequent in B-cell lymphomas, mostly representing aberrant bystanders of physiological rearrangement processes, such as V(D)J recombination or switch recombination (CSR) (reviewed in [2,19]). However, most of these translocations are alone insufficient to drive lymphomagenesis. Additional mutations are usually required for full transformation to malignant disease [2,19]. We therefore propose a multistep model for the lymphomagenesis in our index patient: The t(14q32.33;16;24.1) translocation represents presumably the *initial transforming event* early in the B-cell ontogeny. It affected only one allele without interfering with further B-cell maturation. The other IGH allele rearranged normally. *Additional transforming events* apparently occurred later after antigen contact and GC reaction. Probably only then, the proliferation of the mature translocation carrying B-cells was finally unleashed for the ultimate progression to the diffuse large B-cell lymphoma (**Fig4**).

Taken together, we demonstrate that the combined application of LDI-PCR and interphase FISH represent useful screening tools for the detection of new translocations as well as determination of their incidence in DLBCL on a large patient cohort. However, although IRF8 is widely expressed in DLBCL the mechanism by which it is overexpressed needs still to be elucidated.

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MT, SF and RT established and performed PCR, sequencing and FISH probes and analysed the respective results. SV analysed the immunohistochemical staining and performed statistics. DK collected and analysed survival data. DZ supervised the molecular analysis and wrote the manuscript together with MT.

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Figure legends

Fig 1

Summary of the translocation t(14;16)(q32.33;q24.1) related findings: ***"Chromosomes"*** Chromosomal map illustrating the involved genes and the FISH probes next to the idiograms **"*Restrictions/Primers"*** indicates the restriction sites and primer locations used for the LDI-PCR screening, depicted above the relevant portions of the *IGH* gene. **"*Genes/Fusions*"** *IGH/IRF8 fusion as determined by LDI-PCR and sequencing analysis.* The breakpoint on the *IGH* locus occurs in the joining region near the 5' end of the JH2 element, providing evidence that the translocation occurred during the VDJ rearrangement early during the B-cell maturation. On chromosome 16q24.1 the breakpoint is located about 500 bp upstream of transcription initiation site of the *IRF8* gene, a transcription factor of the interferon (IFN) regulatory factor (IRF) family. Arrows under the gene names indicate the orientation of the coding strand.

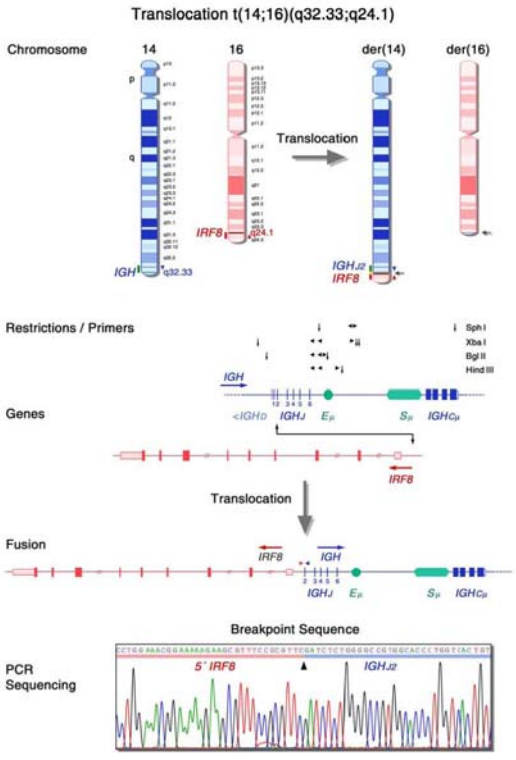

Fig 2

Immunohistolological and in situ hybridisation analyses. **A:** Germinal centre-focused nuclear staining for IRF8 in a reactive tonsil. **B:** Corresponding control staining omitting secondary antibodies. ***C and D:*** Representative examples of IRF8 immunstainings in different DLBCL tissue cores of a TMA demonstrating presence or absence of IRF8 expression, respectively. **E-G:** Interphase FISH with gene probes specific for *IGH* (FITC labelled; green) and *IRF8* (TRITC labelled; red). Cells of the CD5+DLBCL of the index patient showing yellow fusion signals, indicative of a *t(14;16)(q32.33;q24.1)* translocation that joins the *IGH* and *IRF8* loci **(E, F)**. The corresponding FISH staining of a translocation-negative DLBCL displays split signals **(G)**.

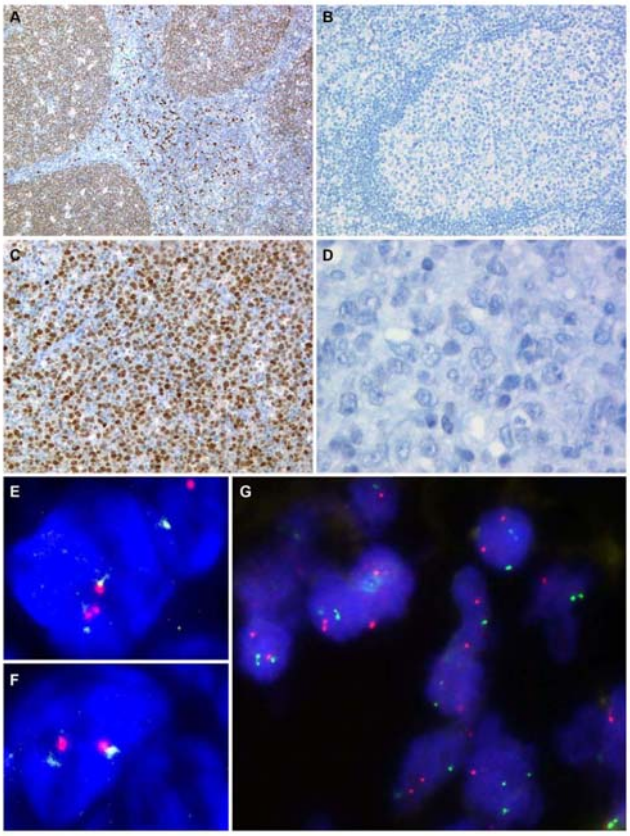

Fig 3

Index patient with DLBCL **A**: Giemsa stain showing an aggressive lymphoma consisting of cohesive sheets of blasts with many mitotic figures. **B**: Membrane positivity for CD5. **C:** double stain for membranous CD20 (red) and nuclear IRF8 (brown) **D:** Interphase FISH with IGH break-apart probe showing one normal allele (green-red fusion = yellow dot) and two split signals (green and red), indicating a break in the IGH locus.

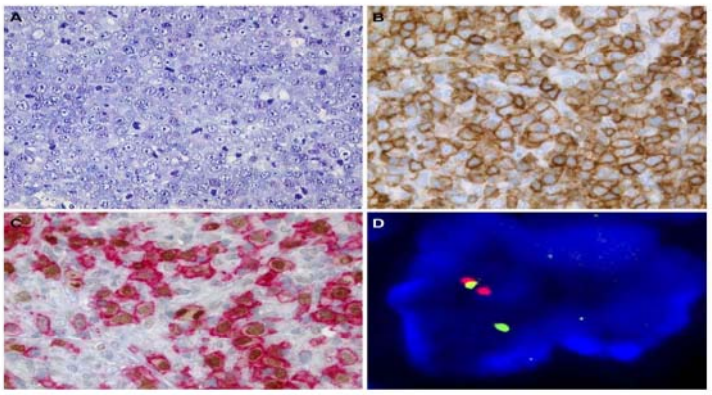

Fig 4

Hypothetical sequence of the multistep transformational process in the index patient. The translocation represents the primary hit in an immature B-cell in the bone marrow followed by an unknown secondary event after germinal centre reaction in the mature B-cell.

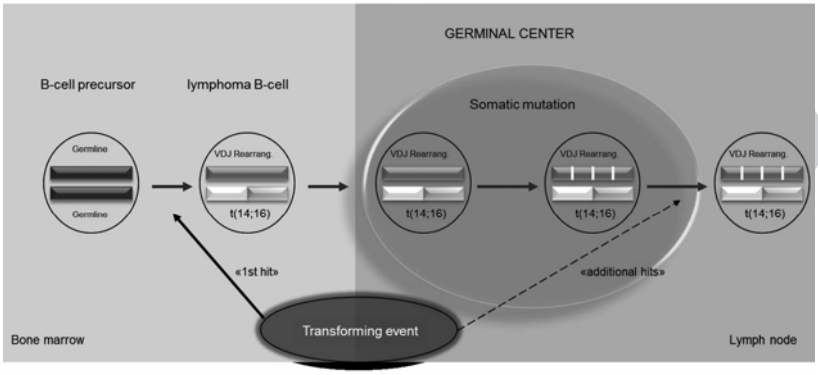

Fig 4

Table Legend

Table 1

Primers for PCR and probes for interphase Fluorescence in Situ Hybridization (FISH)

LDI-PCR: IGH Region primers	
Designation	***Sequence***
Low7836JH6 Low8577Em: Up8716BglII: Up9391HindIII: Up9879Xba: Switch mu upper Switch mu lower	5' TAGCAGAAAACAAAGGCCCTAG AGT 3' 5' CAGACACATATCACTCATGGGT GTT 3' 5' GAAGCTGGAAGCAGATGATGA ATTA 3' 5'
PCR: Translocation-specific primers	
Designation	***Sequence***
Chr 16 JH2	5' AGCCGCTCCTGGGT GG 3' 5'
PCR: IGH-specific primers	
Designation	***Sequence***
FR3 FR2a JH VH4L	5' ACACGGC(C/T)GT(G/A)TATTACTGT 3' 5' TGG(A/G)TCCG(A/C)CAG(C/G)C(C/T)(C/T)C(A/C/G/T)G G 3' 5' ACCTGAGGAGACGGTGACC 3'
FISH assay: BAC clones	
Gene	***Clone name***
IRF8/ICSBP1 IGH (by Chrombios)	RP11-542M13 RP11 - 249 M16 RP11
PCR: BAC clone-specific primers	
Designation	***Sequence***
RP11-542M13 IRF8E8up RP11-542M13 IRF8E8low	5' CTGCTGCTGCGGG AGT 3' 5'